CAMOS, a non-progressive, autosomal recessive, congenital cerebellar ataxia, is caused by a mutant Zinc-Finger protein, ZNF592.

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Abstract

CAMOS (Cerebellar Ataxia with Mental retardation, Optic atrophy and Skin abnormalities) is a rare autosomal recessive syndrome characterized by a non-progressive congenital Cerebellar ataxia associated with Mental retardation, Optic atrophy and Skin abnormalities. Using homozygosity mapping in a large inbred Lebanese Druze family, we previously reported the mapping of the disease gene at chromosome 15q24-q26 to a 3.6cM interval between markers *D15S206* and *D15S199*. Screening of candidate genes lying in this region led to the identification of a homozygous p.Gly1046Arg missense mutation in *ZNF592*, in all five affected individuals of the family. *ZNF592* encodes a 1267-aa zinc finger protein, and the mutation, located within the eleventh zinc finger, is predicted to affect ZNF592's DNA binding properties. Although the precise role of ZNF592 remains to be determined, our results suggest that ZNF592 is implicated in a complex developmental pathway, and that the mutation is likely to disturb the highly orchestrated regulation of genes during cerebellar development, by either disrupting interactions with target DNA or with a partner protein.

Introduction

Non Progressive Congenital ataxias (NPCA) are a small group of disorders, accounting for about 10% of non-progressive infantile encephalopathies [1]. Clinically, NPCA manifests in infancy with abnormal motor development, hypotonia, and delayed ability to sit and stand, followed by the appearance of ataxia [1]. Dysarthria, mental retardation, and spasticity are also often present [2]. Pathologically, most of the congenital ataxias are characterized by hypoplasia or atrophy of parts of the cerebellum and brain stem [3]. It has been estimated that 50% of cases of congenital ataxia and mental retardation are genetically determined, most being of autosomal recessive inheritance [4]. To date, about seventeen loci responsible for NPCA are known, of which fourteen are of autosomal recessive transmission. When excluding the nine loci responsible for Joubert syndrome, five loci are assigned to autosomal recessive NPCA: Cayman ataxia (MIM 601238) at chromosome 19p13.3 [5], due to mutations in *ATCAY* (MIM 608179)[6], Cerebellar ataxia 3 (SCAR6) at chromosome 20q11-q13 (MIM 608029)[7], disequilibrium syndrome at chromosome 9p24 (MIM 224050)[8], "Norman type" ataxia (SCAR2 or CLA1) at chromosome 9q34-9qter (MIM 213200)[9], and CAMOS (SCAR5) at chromosome 15q24-q26[10].

ZNF592 is a 1267-aa zinc finger protein, predicted to contain thirteen classical C2H2-type zinc finger domains. C2H2 Zinc fingers (ZnF) were first discovered in transcription factor IIIA from *Xenopus laevis*[11] and constitute one of the most common DNA-binding motifs found in eukaryotes[12]. C2H2-ZnF proteins usually play a crucial role as transcriptional regulators by mediating interactions between DNAs and proteins [11] and perform various functions in cellular processes, such as transcription, translation, metabolism and signalling by binding to nucleic acids or proteins [13, 14].

In this study, we report the identification of a missense mutation in *ZNF592,* encoding a novel, highly conserved, zinc finger transcription factor, as the pathogenic mutation causing CAMOS, a

rare NPCA syndrome, where Cerebellar Ataxia is associated with Mental Retardation, Optic Atrophy and Skin abnormalities[15].

Material and Methods

Patients

We previously published the localization of CAMOS to chromosome 15q24-q26 in a large consanguineous Lebanese family (Fig 1) [10]. Briefly, all patients share the following clinical features: severely delayed developmental milestones, severe psychomotor retardation, proportionate short stature, cerebellar spastic ataxia, microcephaly, optic atrophy, speech defect, ultrastructural skin abnormalities, and cerebellar atrophy, most probably of prenatal onset. For a fully detailed clinical description of the patients, see the work of Mégarbané et al.[15]. After informed consent was obtained from all individuals and parents of children included in this study, EDTA blood samples were collected and genomic DNA was extracted from lymphocytes using standard methods. All together, fifteen DNA samples were collected for the study, including 5 affected individuals (Fig1). All protocols performed in this study complied with the ethic guidelines of the institutions involved.

Mutation analysis

Of the twenty-four candidate genes present in the 3.6 cM homozygous interval, as described in the databases (Ensembl Genome Browser, UCSC Human Genome Browser), the following ten were selected, based on their function and tissue-specific expression; and tested for the presence of a pathogenic mutation: *AP3B2, SH3GL3, BTBD1, HMG20A, HOMER2, RKHD3, EFTUD1, ZSCAN2, SCAND2* and *ZNF592* (Fig 2A). Exploration of the entire coding sequence, as well as of exon-intron boundaries and 5' and 3' untranslated regions (UTRs), was performed for each candidate gene (Fig 2A)**.** Intronic primers were designed using the Primer3 and OLIGOS v.9.3 softwares. Primer sequences and annealing temperatures used for PCRs are available as supplementary material (Table 1). DNA sequences were obtained from the UCSC Human

Genome Browser (UCSC, March 2006 freeze), by comparing genomic DNA with cDNA sequences. Genomic DNA from Patient VI.14 and a control were amplified under standard PCR conditions. All PCR amplified fragments were analyzed by dHPLC/WAVE (Transgenomic, Omaha, NE, USA) and fluorescently sequenced in both directions, using an ABI 310 or ABI 3130 automated sequencer (Applied Biosystems, Foster City, USA), for those presenting abnormal elution profiles (conditions for dHPLC analysis are available upon request). Chromatograms were compared to reference sequences using Sequencher v4.2 (Gene Codes Corporation, Michigan, USA) and ChromasPro v1.33 (Technelysium, Tewantin, Australy).

Segregation in the pedigree, of the identified c.3136G>A nucleotide change, as well as its presence in 444 Lebanese control chromosomes, were assessed by PstI restriction endonuclease digestion (Boehringner Mannheim) or dHPLC.

Transcriptional analysis

Total RNA was extracted from freshly isolated lymphocytes or immortalized lymphocytes, using standard protocols as described before [16]. cDNAs were obtained from total RNA with use of Superscript I Reverse Transcriptase (Invitrogen Life Technologies) and random primers (InvitrogenLife Technologies), in accordance with the recommendations of the supplier. cDNAs were then PCR amplified as described above. Primers and annealing temperatures for amplification of the whole *ZNF592* cDNA are available as supplementary material (Table 1). Primers corresponding to fragment 6 have been used for verification of the effect of the variation on *ZNF592's* splicing.

Semi-quantitative RT-PCR

In human adult tissues, *ZNF592's* expression profile was characterized, using Rapid Scan Gene Expression Panels from ORIGENE (Rockville, USA). These panels consist of cDNAs synthesized

from poly(A+) RNA and immobilized at four cDNAs concentrations (100X and 1000X) on a 96-well PCR plate. Human panels include 24 different human adult tissues and 12 brain tissues (Supp. fig.1A-B). Expression of *ZNF592* during development was assessed in 6 human foetal tissues by semi-quantitative RT-PCR using Multiple Choice cDNAs from ORIGENE (Rockville, USA), and, in mouse, using mRNAs extracted from whole embryo*,* at different developmental stages (Supp. fig.1D-E).

Primers used for amplification are listed in Table 1 (Supplementary Material). In all experiments, *ß*-Actin was used for normalisation and amplified using primers included in the kit (ORIGENE, Rockville, USA).

Northern-Blot

Quantitative Northern-Blot analysis of *ZNF592* expression in different human tissues was realized using commercially available synthetic polyA+ membranes from Ambion (Foster City, USA) and Clontech (Moutain View, USA), that were hybridized with a 972 bp radioactively labelled ([^{32}P]-dATP) cDNA probe, covering *ZNF592* exons 3 to 8. A Human *ß*-Actin cDNA probe provided by the manufacturer (ORIGENE, Rockville, USA) was used as a normalizing control.

For patients' Northern-Blot, we immobilized polyA+ mRNAs, extracted from homozygous patient VI.1 's, heterozygous father V.2's and Lebanese control's lymphoblastoid cells, using the µMacs mRNA isolation kit from Myltenyibiotech (Bergisch Gladbach, Germany); and the obtained synthetic membrane was hybridized to the 972 bp *ZNF592* cDNA probe cited above. All steps were realized using the NorthernMax-Gly kit (Ambion, Foster City, USA), following the manufacturer's recommendations.

Real-Time RT-PCR (QRT-PCR)

SYBR Green real-time PCRs (QRT-PCRs) were realized according to standard protocols in a 20-µl amplification mixture containing 10 µl of SYBR® Green PCR Master Mix buffer (Applied Biosystems, Foster City, CA, USA), 0.25 µM of each primer and 20 ng of RNAse H treated (Invitrogen Life Technologies) cDNAs obtained from reverse transcription of 5µg of DNAse I–treated total RNA extracted from homozygous patient VI.1, heterozygous father V.2 and controls blood lymphocytes by use of the High Capacity cDNA Archive Kit (Applied Biosystems). Reactions were performed and data collected on the ABI PRISM 7500 Sequence Detection System (Applied Biosystems, Foster City, CA, USA) using conditions recommended by the manufacturer. Each assay included non-template controls and the unknown samples in triplicates: i.e patient VI.1, heterozygous father V.2. For *ZNF592*, five different calibrators (Lebanese controls) were used. All experiments have been repeated twice, with a freshly extracted mRNA from patient VI.1 and father V.2. The expression values of *ZNF592* were normalized to that of *GUS* and *GAPDH,* while *CCDN1 and CCDN2* expression values were normalized to that of *TBP* and *GUS*. For each gene, the expression ratio for a sample was calculated as the ratio between the average *mRNA* signal in the patient and the signal from calibrators. After the reaction, the data were analyzed using the comparative *C*T relative quantification method with ABI PRISM 7500 system SDS software version 1.2.3 (Applied Biosystems, Foster City, CA, USA). Primers for QRT-PCR were designed using Primer Express software version 2 (Applied Biosystems, FosterCity, CA, USA). All primer sequences are listed in Table 1 (Supplementary Material). Results are presented as average values for experiments with *GUS* as a normalizing gene.

Protein homology modelling studies

Wild-type and mutant eleventh ZNF592's C2H2 Zinc Finger (ZnF) were modelized using freely available Swiss-Pdb Viewer software. As no PDB structure was available for ZNF592 in the

Protein Data Bank (PDB), we used the structure of the C2H2 zinc-binding domain from ZFP64/ZNF338 (PDB ID : 1X5W), another human zinc finger protein, presenting sequence homologies with ZNF592's zinc fingers. Using Swiss-Pdb Viewer, we changed all amino acids (aa) to ZNF592 aa. In the mutant ZnF, the glycine at position 41 was replaced by an arginine, as observed in patients affected with CAMOS, who harbour the p.Gly1046Arg missense mutation. In the mutant C2H2 zinc-finger domain 3D structure (B), mutation of Gly41 to Arg41 results in the formation of a new hydrogen bond interaction between Arg41 and the adjacent tyrosine Tyr42.

Web resources

GenBank Accession numbers:

AP3B2: NM_004644

SH3GL3: NM_003027

BTBD1: NM_025238

HMG20A: NM_018200

HOMER2: NM_199330

RKHD3: NM_032246

EFTUD1: NM_024580

ZSCAN2: NM_181877

SCAND2 : NM_033634 (the sequence was suppressed afterwards as the transcript is in fact a NMD transcript)

ZNF592: BC094688, NM_014630, NC_000015, ENST00000299927, ENSG00000166716

In the databases, discrepancies exist between GenBank (NM_014630 and NC_000015), the UCSC Genome Browser, and the Ensembl browser (ENST00000299927, ENSG00000166716) concerning the length of the transcript (but not the CDS), the number of exons, the length of the

UTRs and the region covered by *ZNF592*. Based on results of Northern blot experiments (see Suppl. Fig 1C), we used the sequence with Acc. Number BC094688, as a reference in our study.

ZFP64/ZNF388: NP_060667

PDB ID for ZFP64/ZNF338: 1X5W

ZNF592 proteins: NP_055445 (*Homo sapiens*), NP_848822 (*Mus musculus*), XP_001084237 (*Macaca Mulatta*), NP_001099742 (*Rattus norvegicus*), XP_545869 (*Canis familiaris*), XP_001916274 (*Equus Caballus*), NP_001095506 *(Bos Taurus),* XP_001364673 (*Monodelphis domestica*), NP_001072160 (*Xenopus Tropicalis*), NP_001038678 (*Danio Rerio*).

URLs:

Online Mendelian Inheritance in Man (OMIM): http://www.ncbi.nlm.nih.gov/Omim

Design of primers available at http://frodo.wi.mit.edu/

The UCSC Genome Browser is available at http://genome.ucsc.edu/cgi-bin/hgGateway.

Ensembl Server: http://www.ensembl.org/

NCBI dbSNP database: http://www.ncbi.nlm.nih.gov/SNP/

RCSB Protein Databank: http://www.rcsb.org/pdb/home/home.do

Swiss-Pdb Viewer: http://spdbv.vital-it.ch/

DEPP (Disorder Enhanced Phosphorylation predictor):

http://www.pondr.com/Phosphorylation_PONDR

Results

Using DNA pooling and homozygosity mapping, we had previously assigned CAMOS to a 3.6 cM (5.7 Mb) homozygous region at chromosome 15q24-q26 between markers *D15S206* and *D15S199*, in a large consanguineous Lebanese family, with five affected individuals [10] (Fig 1). Screening of the coding sequence of genes lying in the candidate interval, by direct sequencing, allowed us to identify a c.3136G>A homozygous transition in exon 6 of *ZNF592*, a gene encoding a 1267-aa zinc finger protein, and predicted to contain thirteen classical C2H2-type zinc finger domains (Fig 2). The c.3136G>A nucleotide change identified in our patients was not reported in the NCBI dbSNP database, segregated perfectly with the phenotype in the pedigree and was not found in the 444 tested Lebanese control chromosomes, excluding the possibility that it might be a rare polymorphism and indicating that this variant was likely to be pathogenic. As the variation is affecting the second last nucleotide of exon 6, just upstream the 5' consensus donor splice site, we tested whether it might affect splicing of *ZNF592* transcript. RT-PCR studies using total RNAs extracted from patients and Lebanese control's blood lymphocytes, and primers located in exons surrounding *ZNF592* exon 6, allowed us to rule out this hypothesis, at least in the tested tissue (Fig 2C). Occurrence of small deletions/insertions was excluded by sequencing of the obtained RT-PCR fragment in patients and control. Other complex splicing defects were excluded by amplification and sequencing of the entire cDNA. At the protein level, the *ZNF592* c.3136G>A mutation is likely to be a missense mutation, leading to the replacement of a glycine by an arginine at residue 1046 (p.Gly1046Arg; GGG>AGG). It targets a highly conserved amino acid residue among eutherian mammals, located in the eleventh C2H2 zinc-finger domain, at the base of the loop between the two conserved cysteines (Fig 3).

Protein homology modelling using wild-type and mutant predicted C2H2 zinc-finger domain 3D structure showed that mutation of glycine to arginine results in the formation of a new

hydrogen bond interaction between Arg41 and the adjacent tyrosine Tyr42 in the mutant C2H2 zinc-finger (Fig 4).

Considering the specificity of tissues involved in our pathology (CNS and skin), we characterized *ZNF592's* expression profile, in both human and mouse tissues (Supp. fig. 1). Both semi-quantitative RT-PCR experiments, using Rapid Scan Gene Expression Panels from ORIGENE (Rockville, USA), and Northern Blot experiments demonstrated ubiquitous expression of *ZNF592* in all studied human adult tissues, notably in brain and skin, with higher expression in skeletal muscle (Supp. fig 1A-C). In the Central Nervous System (CNS), *ZNF592* is expressed widely, including in cerebellum and cerebellar vermis, with higher expression in Substantia Nigra (Supp. fig 1B). Very low levels of *ZNF592* mRNA were detected in ovary, uterus and salivary glands (Supp. fig 1C). Finally, semi-quantitative RT-PCR experiments showed expression of *ZNF592* in six tested human foetal tissues and early expression of *Zfp592 (ZNF592 m*ouse ortholog) during development: as early as embryonic day E10 in whole mouse embryo (Supp. fig 1D-E). In mouse brain, *Zfp592* mRNAs were detectable at birth (P0) and adult stages (Supp. fig 1E).

In patients affected with CAMOS, we analysed *ZNF592*'s mRNA levels using Northern Blot and Quantitative Real-Time RT-PCR (QRT-PCR). Both experiments evidenced higher levels of *ZNF592* transcript levels in both the patient, and heterozygous father, as compared to control (data not shown for Northern Blot and Supplementary figure 2 for QRT-PCR), with a mean 3-fold increase in patient VI.1. Surprisingly, *ZNF592* transcripts levels were even higher in the heterozygous father. No statistical analysis could be performed due to the small size of the sample (only one patient), however all experiments have been performed in triplicates and have been repeated twice, with freshly extracted total RNA samples in the second experiment. Unfortunately, we were not able to confirm these results at the protein level, as a polyclonal antibody against ZNF592 generated in rabbit failed to detect ZNF592 in control tissues (data not shown).

We studied the expression of cyclin-D1 (*CCND1*) and -D2 (*CCND2*) in CAMOS patient VI.1 's lymphocytes using QRT-PCR. Interestingly, we found an increase in *CCND1* and *CCND2* mRNA levels; again, both in the patient and in the heterozygous father as compared to control, with a mean 43-fold and 3-fold increase in *CCND1* and *CCND2* patient's transcripts levels respectively. As for *ZNF592*, no statistical analysis could be performed, but all experiments have been performed in triplicates and have been repeated twice.

Discussion

CAMO is a rare non progressive cerebellar ataxia syndrome, where Cerebellar Ataxia is associated with Mental Retardation, Optic Atrophy and Skin abnormalities[15]. Since the first report and localization of the gene at chromosome 15q24-q26 in a large consanguineous Lebanese family [10,15], no additional patients have been reported, pointing out the very low prevalence of this disease. In this study, we report the identification of a c.3136G>A missense mutation (p.Gly1046Arg) in *ZNF592,* in patients from this family. ZNF592 is a novel, highly conserved, 1267-aa zinc finger protein, predicted to contain thirteen classical C2H2-type zinc finger domains (ZnF) (Fig 2A and 3A), which constitute one of the most common DNA-binding motifs found in eukaryotes[12]. Several genes encoding zinc-finger proteins have been implicated in human pathologies, such as *ZNF215* in Beckwith-Wiedemann syndrome[17] (MIM 130650); *ZNF41* (MIM 314995)[18], *ZNF81*[19] (MIM 314998) and *ZNF674*[20] (MIM 300573) in non syndromic, X-linked mental retardation or *ZNF750* in Seborrhea-like dermatitis with psoriasiform elements[21]. *ZNF592* is the first gene of this family found to be mutated in a cerebellar syndrome, although mutations have been described in *APTX* (MIM 606350), the gene mutated in oculomotor-apraxia (AOA1) and encoding aprataxin, a nuclear protein with a role in DNA repair and containing one C2H2 ZnF[22,23].

The p.Gly1046Arg mutation identified here targets a highly conserved amino acid and is located within the eleventh zinc finger of ZNF592. *In silico* protein homology modelling studies predict a destabilizing effect for the mutation, via the formation of a new hydrogen bond interaction between the mutated arginine and the adjacent Tyrosine (Fig 4), thereby leading to the disruption of the ZnF, as described for other transcription factors [24,25,26]. By affecting ZNF592's DNA binding properties or interactions with other TFs, the p.Gly1046Arg mutation in *ZNF592* might also regulate *ZNF592*'s own expression, as evidenced by the higher levels of *ZNF592* transcripts observed in the studied patient homozygous for the p.Gly1046Arg mutation. Thus, the ratio of wild-type versus mutant ZNF592 might be crucial, explaining the peculiar pattern of expression observed in heterozygote father versus homozygote patient.

The multiplicity of tissues/organs involved, as well as the non-progressive nature of CAMOS, is characteristic of a developmental disease, with abnormal development of the cerebellum as a hallmark. Several genes for congenital human cerebellar malformations have been identified to date, including members of the Zic family of C2H2 zinc finger TFs[27]. Indeed, heterozygous deletions of the adjacent *ZIC1* (MIM 600470) and *ZIC4* (MIM 608948) genes cause Dandy-Walker malformation (MIM 220200), the most common human congenital cerebellar malformation[27], and deletion of *Zic1* in mouse results in cerebellar malformations and axial skeletal abnormalities[28]. Zic genes play critical roles in a variety of developmental processes and are regulated by a number of pathways/TFs. We postulate that ZNF592 might be a downstream or upstream element in the Zic regulated pathways. By disrupting the interactions between ZNF592 and its target consensus sequence, or between ZNF592 TF and another TF (as described for Zic with members of the Gli family of C2H2 TFs[29,30]), the p.Gly1046Arg mutation identified here is likely to affect the pathways regulated by Zic genes. This hypothesis is supported by the early expression of *ZNF592*, as early as E10 (Supp. fig 1E), the non progressive nature of CAMOS, and the presence of a dilated fourth ventricle in CAMOS patients as observed in patients

affected with Dandy-Walker malformation. Cyclin D1 (MIM 168461), a downstream effector of Zic proteins[31] , shows reduced transcript expression levels in the cerebella of Zic mutants[32], and mice lacking cyclin D1 and cyclin D2 (MIM 123833) (cyclin D3-only mice) lacked normally developed cerebella[33]. In our patient's lymphocytes, we observe an increase in *cyclin-D1* (*CCND1*) and -*D2* (*CCND2*) mRNA levels, especially important for *CCND1* (43 fold increase). Although preliminary, these results of altered expression patterns are extremely interesting and bring further support to the hypothesis of ZNF592, as a TF implicated in a complex developmental pathway. Indeed, D1-cyclin has been shown to be upregulated by Notch signalling[34], known to inhibit differentiation of cerebellar granule neuron precursors by maintaining proliferation[35]. Abnormally elevated levels of cyclin D1 in cerebellar granule neuron precursors, by disrupting proliferation/differentiation processes in these cells, might have impaired cerebellar development in our patients. In this context, the surprisingly elevated levels of *ZNF592*, *cyclin D1* and *D2* transcripts in the heterozygous father (Supp. fig. 2), are difficult to interpretate, but might be explained by the fact that the regulation of the described pathways is highly orchestrated in time, and that the amount of each TF might change rapidly in time, with a combination of tightly regulated levels of expression.

In conclusion, this study describes the identification of *ZNF592* as the gene responsible for CAMOS, a rare, non progressive, cerebellar ataxia syndrome previously identified in a Lebanese family. Further studies have to be performed in order to elucidate the pathophysiological mechanisms underlying CAMOS. However, our results allow us to formulate the hypothesis that ZNF592 might be implicated in a complex developmental pathway, affecting several tissues, such as the "Zic/Cyclin/Notch" pathway. A major argument in favour of the involvement of ZNF592 in this pathway is the presence, in our patients, of an inversion of the usual osmiophilic pattern of the vessels in skin biopsies: the endothelial cells in patients are osmiophilic, compared to normal endothelium, which is electrolucent[15]. Indeed, neural and vascular

precursors use common signals and pathway, such as Notch, Sonic Hedgehog (Shh) or BMP, to specify their fate; and neural and vascular cells influence the cell- fate decision making of one another[36]. The Notch pathway, in particular, has a crucial role in arterial Endothelial Cell (EC) specification, by inducing arterial fate at the expense of venous EC fate[36]. Implication of ZNF592 in a pathway common to neural and vascular progenitors would explain the affection of both tissues in CAMOS.

The identification of ZNF592 as the defective protein in CAMOS constitutes a first step in the comprehension of the pathophysiological mechanisms underlying this syndrome. Further experiments will be conducted in order to better define which pathways are involved.

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Supplementary information is available at the European Journal of Human Genetics' website

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Legends to figures and tables

Figure 1. Pedigree of the Lebanese family

Blackened symbols indicate the affected individuals. Gray circles indicate patients affected with diastrophic dysplasia, another autosomal recessive disease segregating in this pedigree.

Figure 2. Candidate genes in the homozygous region identified in CAMOS with, identification of mutations in *ZNF592*.

(A) Chromosomal region at 15q24-q26 containing the CAMOS candidate locus as previously identified[10], covering a 5.7 Mb (3.6 cM) region located between STR markers D15S206 (AFM299yf9) and D15S199 (AFM107xg7). The 24 known genes lying in this region are schematically represented, with candidate genes tested in our study indicated in blue. *ZNF592* is represented in red. *ZNF592* is located at chromosome 15q25.3, covering a genomic region of about 20 kb, and is transcribed in direction centromere to telomere, as indicated by the black arrow. In *ZNF592* 's transcript, coding exons are represented in red and pink. *ZNF592* is composed of 8 exons and the transcript is 4312 bp long (Acc. Number BC094688), with a coding sequence of 3804 bp (exons 1-8).In the protein ZNF592, the thirteen C2H2 Zinc Finger (ZnF) domains are represented by boxes. Location of the mutation identified in this study is indicated by an asterix in exon 6 of the gene, or in the eleventh zinc finger of the protein.

(B) Chromatograms showing the c.3136G>A mutation identified in *ZNF592* exon 6 in the Lebanese patients. Lebanese control sequence is shown in a control.

(C) Effect of the mutation on the trancription: agarose gel migration showing a transcript of normal size in the patients. Chromatograms showing the absence of small deletions/insertions and the c.3136G>A missense mutation giving rise to the replacement of a glycine by an arginine in the protein (p.Gly1046Arg).

Figure 3. Schematic representation of the zinc fingers (ZnF) and conservation between species

(A) Schematic representation of ZnFs ten and eleven, showing the location of the mutated glycine at residue 1046, at the base of the 11th ZnF. **(B)** Multiple alignment between human ZNF592 protein and several orthologs. The glycine at residue 1046 mutated in our study is indicated in red and is highly conserved among eutherian mammals

Figure 4. 3D structures of wild-type and mutant ZNF592 C2H2 Zinc finger

Wild-type **(A)** and mutant **(B)** eleventh C2H2 Zinc Finger (ZnF) modeling using freely available Swiss-Pdb Viewer software. In the mutant C2H2 zinc-finger domain 3D structure **(B)**, mutation of Gly41 to Arg41 results in the formation of a new hydrogen bond interaction between Arg41 and the adjacent tyrosine Tyr42.

Legends to supplementary tables and figures

Supplementary Figure 1. Expression Analysis

(A) Semi quantitative expression of *ZNF592* in 24 different human adult tissues.

cDNAs synthesized from poly(A+) RNA and immobilized at four cDNAs concentrations (1X, 10X, 100X and 1000X) on a 96-well PCR plate, were amplified using primers located in *ZNF592* exons 1 and 2 (see Supp. table 1).

(B) Semi quantitative expression of *ZNF592* in 12 different parts of the central nervous system, using immobilized cDNAs at two concentrations as in (A). *ZNF592* is expressed widely, including in cerebellum and cerebellar vermis, with higher expression in Substantia Nigra.

(C) Quantitative Northern-Blot analysis of *ZNF592* expression in different human tissues.

(D) Expression of *ZNF592* in human foetal tissues. NTC: non template control.

(E) Expression of mouse *Zfp592* at different developmental stages.

Whole mouse embryo cDNAs at different embryonic days. E 10: embryonic day 10. E12: embryonic day 12. E15: embryonic day 15. E18: embryonic day 18. P0 brain: mouse brain at birth. Adult brain: mouse adult brain. NTC: non template control.

Supplementary Figure 2. Quantitative Real time RT-PCR (QRT-PCR)

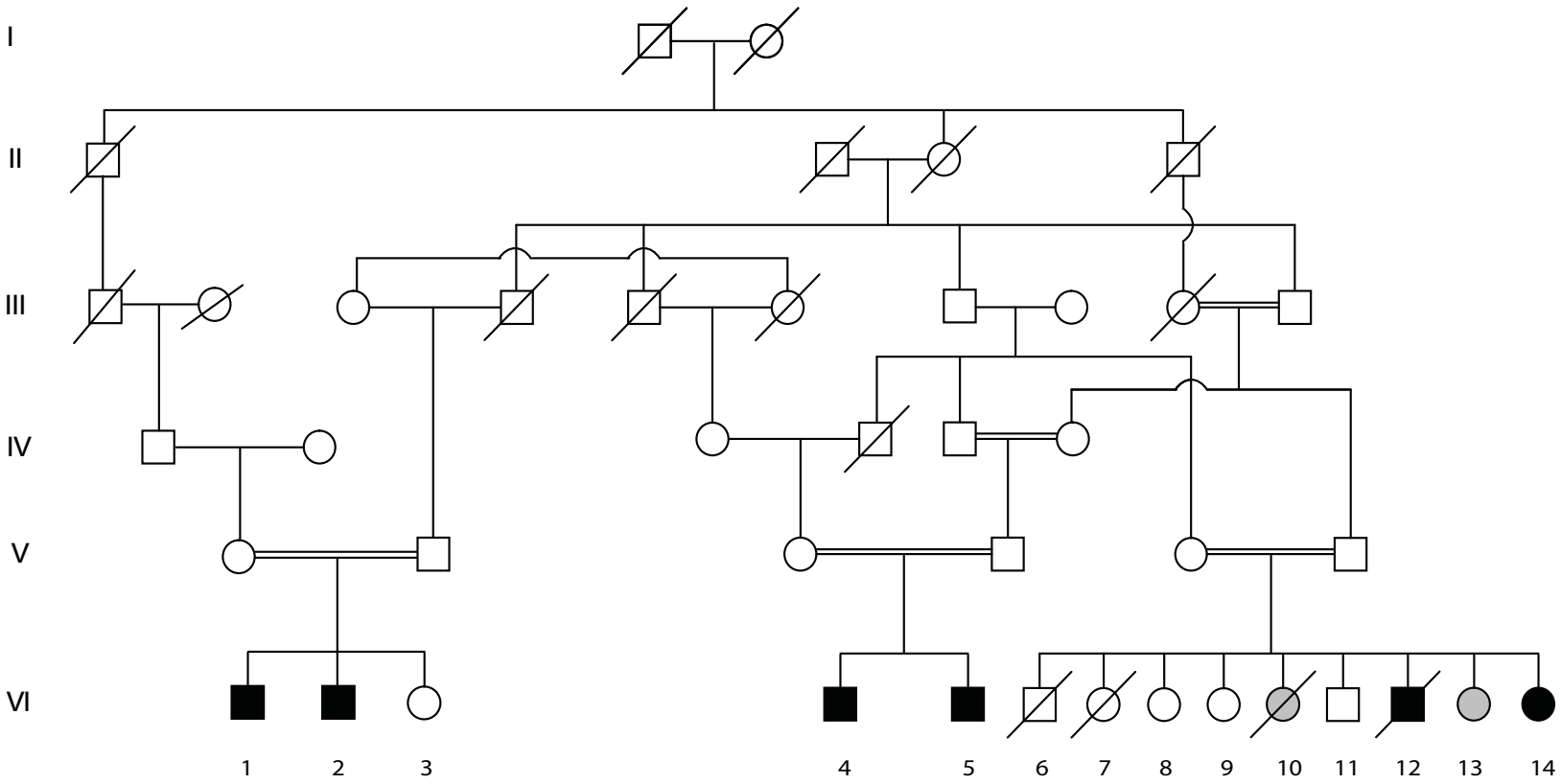
(A) Quantification of *ZNF592* transcripts' levels in patients affected with CAMOS by QRT-PCR.

(B) Quantification of *cyclin-D1 and -D2* transcripts levels in patients affected with CAMOS by QRT-PCR.

Results are presented as average values, with *GUS* as the normalization gene. Het: father V.2. Hom: patient VI.1.

Supplementary Table 1.

Primers sequences and annealing temperatures used for the analysis of *ZNF592* coding sequence at the genomic and transcriptional level. Specific primers for real time RT-PCR are also mentioned. All human primers for genomic screening of mutations have been selected based on comparison of genomic DNA sequence with Genbank cDNA sequence BC094688.


I
II
III
IV
V
VI
1
2
3
4
5
6
7
8
9
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12
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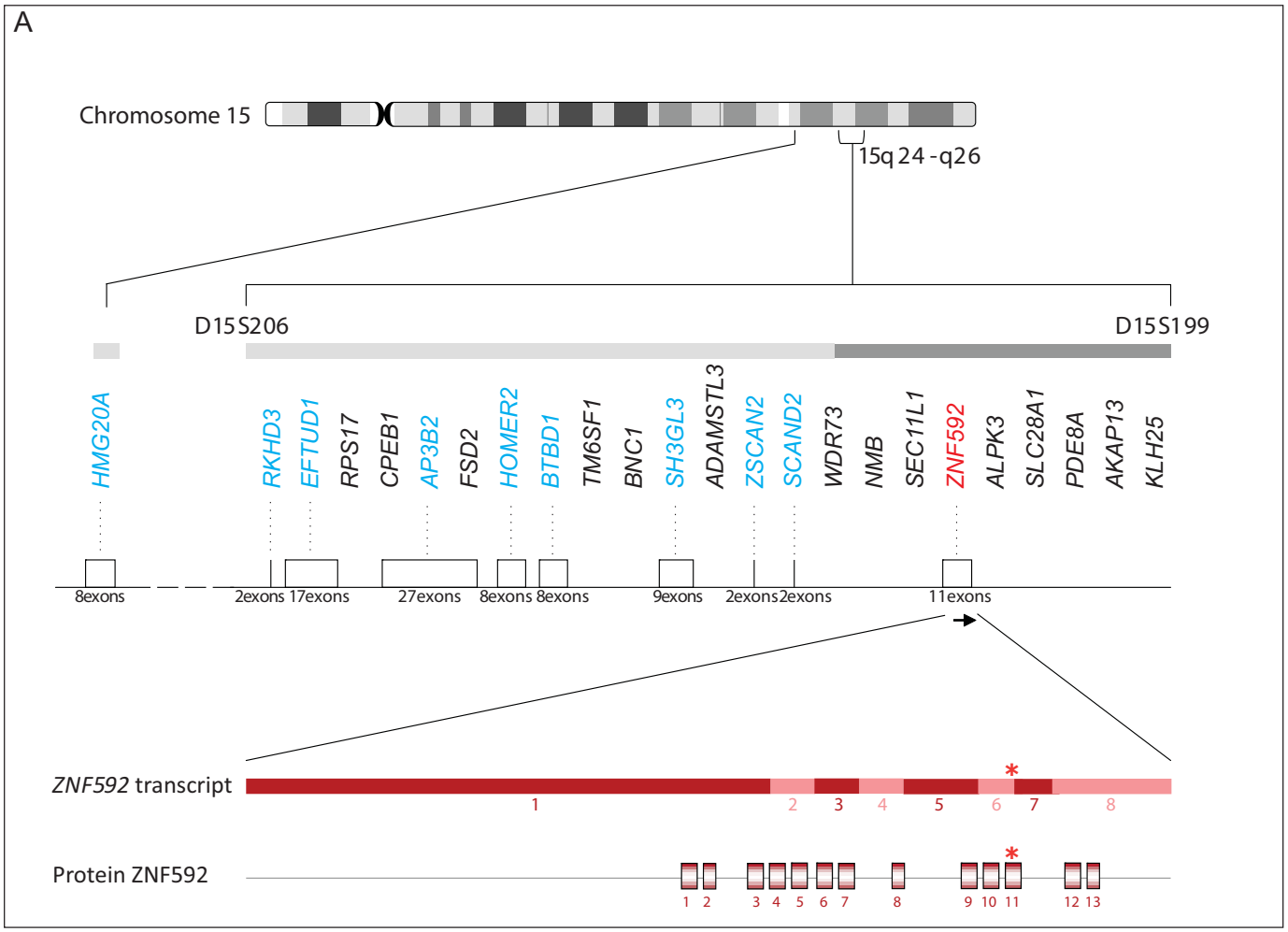
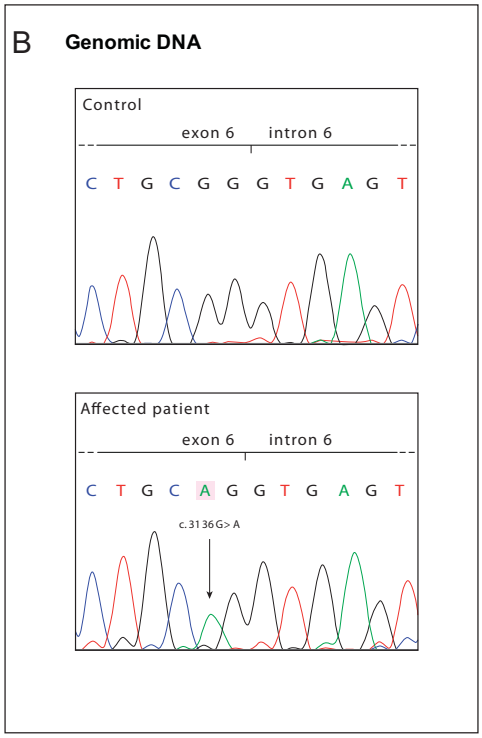
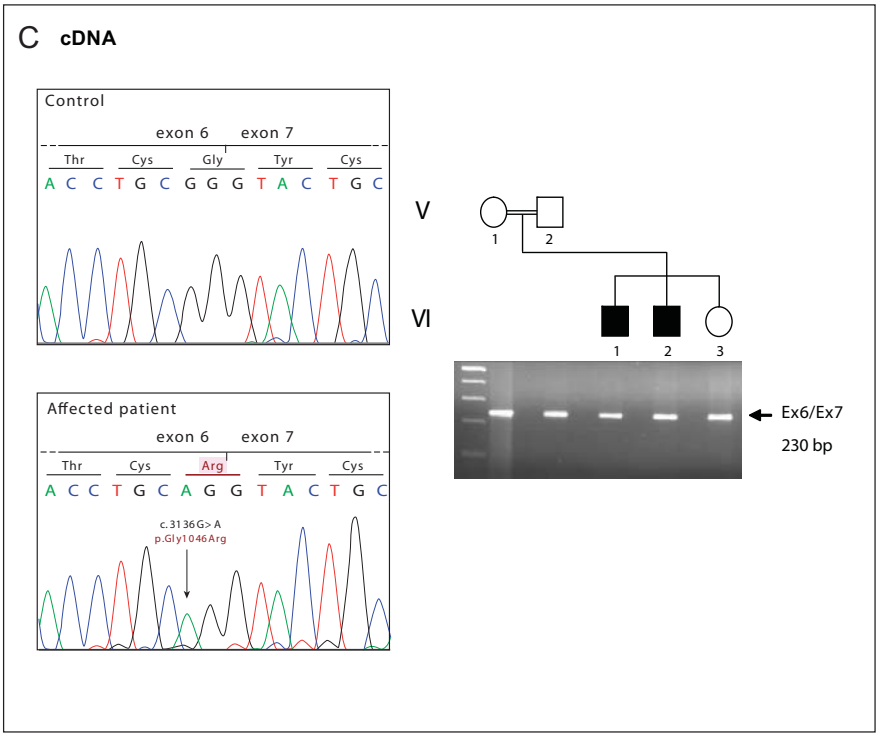
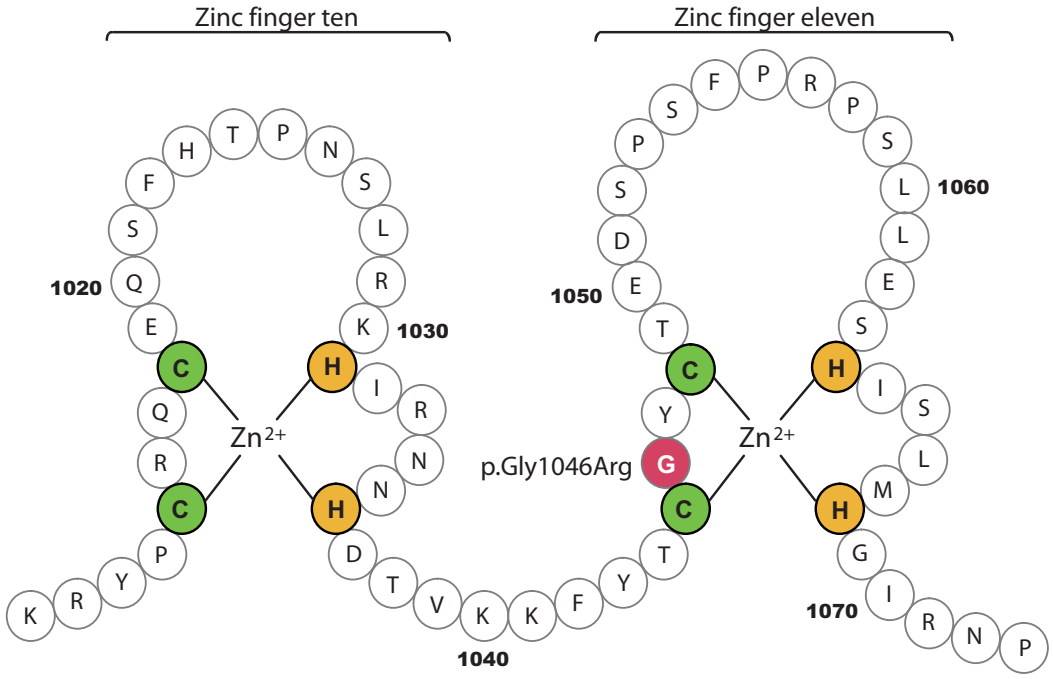
14


A
Chromosome 15
15q24-q26
D15S206
D15S199
HMG20A
RKHD3
EFTUD1
RPS17
CPEB1
AP3B2
FSD2
HOMER2
BTBD1
TM6SF1
BNC1
SH3GL3
ADAMSTL3
ZSCAN2
SCAND2
WDR73
NMB
SEC11L1
ZNF592
ALPK3
SLC28A1
PDE8A
AKAP13
KLH25
8exons
2exons 17exons
27exons
8exons 8exons
9exons
2exons 2exons
11exons
ZNF592 transcript
1 2 3 4 5 6 7 8
Protein ZNF592
1 2 3 4 5 6 7 8 9 10 11 12 13

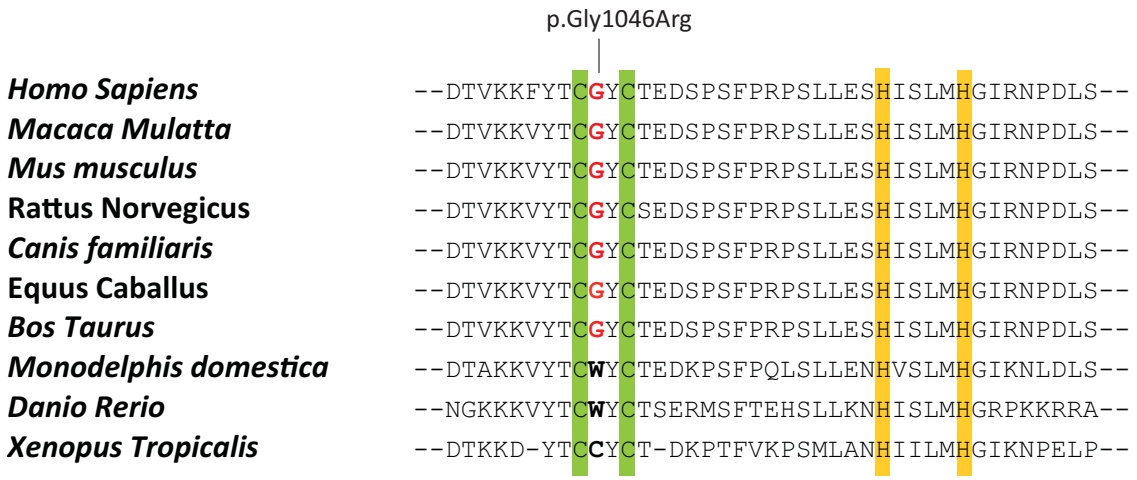

B
Genomic DNA
Control
exon 6 intron 6
C T G C G G G T G A G T
Affected patient
exon 6 intron 6
C T G C A G G T G A G T
c.3136G>A

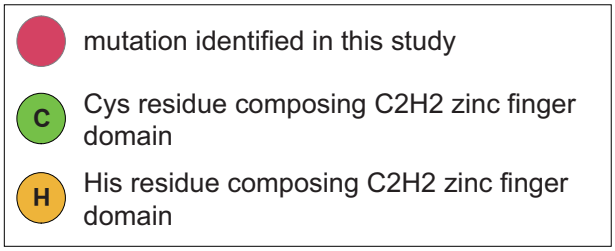

C
cDNA
Control
exon 6 exon 7
Thr Cys Gly Tyr Cys
A C C T G C G G G T A C T G C
Affected patient
exon 6 exon 7
Thr Cys Arg Tyr Cys
A C C T G C A G G T A C T G C
c.3136G>A
p.Gly1046Arg
V
VI
1 2
1 2 3
Ex6/Ex7
230 bp

A

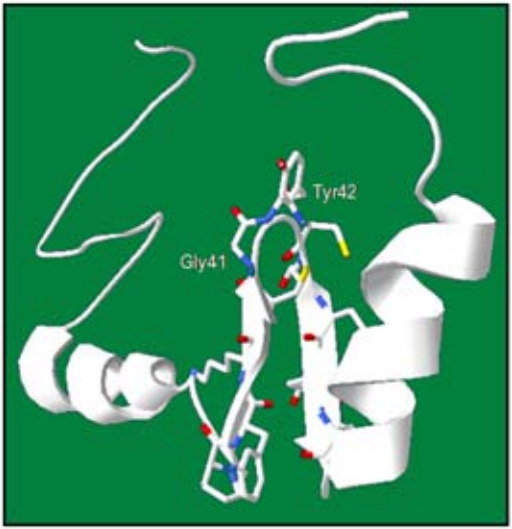

Zinc finger ten
Zinc finger eleven
1020
1030
1040
1050
1060
1070
Zn2+
Zn2+
p.Gly1046Arg

B


p.Gly1046Arg
Homo Sapiens --DTVKKFYTCGYCTEDSPSFPRPSLLESHISLMHGIRNPDLS--
Macaca Mulatta --DTVKKVYTCGYCTEDSPSFPRPSLLESHISLMHGIRNPDLS--
Mus musculus --DTVKKVYTCGYCTEDSPSFPRPSLLESHISLMHGIRNPDLS--
Rattus Norvegicus --DTVKKVYTCGYCSEDSPSFPRPSLLESHISLMHGIRNPDLS--
Canis familiaris --DTVKKVYTCGYCTEDSPSFPRPSLLESHISLMHGIRNPDLS--
Equus Caballus --DTVKKVYTCGYCTEDSPSFPRPSLLESHISLMHGIRNPDLS--
Bos Taurus --DTVKKVYTCGYCTEDSPSFPRPSLLESHISLMHGIRNPDLS--
Monodelphis domestica --DTAKKVYTCWYCTEDKPSFPQLSLLENHVSLMHGIKNLDLS--
Danio Rerio --NGKKKVYTCWYCTSERMSFTEHSLLKNHISLMHGRPKKRRA--
Xenopus Tropicalis --DTKKD-YTCCYCT-DKPTFVKPSMLANHIILMHGIKNPELP--


mutation identified in this study
C
Cys residue composing C2H2 zinc finger domain
H
His residue composing C2H2 zinc finger domain

A



B